IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

FELD et al

Serial No.: 09/691,889

Filed: October 20, 2000

Group Art Unit: 1632

For: Nucleic Acid Constructs and Cells, and Methods Utilizing Same for Modifying the Electrochemical Conductance of Excitable Tissues

Attorney Docket: 00/20989

Examiner: Anne-Marie Falk

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.131

Sir:

I, Yair Feld, declare as follows:

1. I am a co-inventor of the invention described and claimed in the above-identified U.S. Patent Application.
2. I am familiar with the U.S. Patent & Trademark Office Action dated July 29, 2005 on the above identified application, in which the Examiner cited Donohue et al, U.S. Patent Application 2004/0266717 as part of a rejection of my claimed invention under 35 U.S.C. 103.
3. That the effective filing date of the Donohue et al reference is the date of the priority Provisional patent application Serial No. 60/230,311, filed September 6, 2000.
4. That the aforementioned reference to Donohue et al is not prior art to our invention, inasmuch as we had reduced to practice and thus made our invention prior to the effective September 6, 2000 filing date of Donohue et al.

Y.F

5. In evidence of such reduction to practice, I attach herewith

A. A copy of my Ph.D. research proposal (in Hebrew), Exhibit A, submitted to the Technion Institute of Technology prior to September 6, 2000,

B. A letter (Exhibit B) from said Technion Institute indicating receipt thereof prior to September 6, 2000 (date blacked out).

C. A translation of the said Ph.D. research proposal into the English language, (Exhibit C). Figure 4, Paragraph 2 of the Preliminary Results Section of said Ph.D. research proposal, shows that the above identified disclosed and claimed invention was reduced to practice prior to September 6, 2000.

D. A Declaration from Gal Ehrlich, a registered Israeli patent attorney stating that he received and understood the above invention when said research proposal was presented to him prior to September 6, 2000, and that he was instructed by Dr. Shmuel Liran, the authorized official of the Technion, in an email dated prior to September 6, 2000 (Exhibit D, date blackened out) to prepare and file a patent application according to the disclosure received from the inventors, which consisted of said Ph.D. research proposal.

6. That the rejection of claims of our invention over any rejection that employs Donohue et al should be withdrawn since Donohue et al is not prior art relative to the invention that is the subject of the above-identified patent application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Y.F

Respectfully submitted,

Yair Feld

Date: October 18, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		§	
		§	
	FELD et al	§	
		§	
Serial No.:	09/691,889	§	
		§	
Filed:	October 20, 2000	§	Group Art Unit: 1632
		§	
For:	Nucleic Acid Constructs and Cells, and Methods Utilizing Same for Modifying the Electrochemical Conductance of Excitable Tissues	§	Attorney Docket: 00/20989
Examiner:	Anne-Marie Falk	§	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPORTING DECLARATION UNDER 37 CFR 1.131

Sir:

I, Gal Ehrlich, declare as follows:

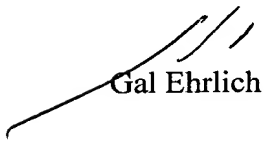
1. I am a patent attorney licensed to practice before the Israeli Patent Office.
2. I am thoroughly familiar with the invention described and claimed in the above-identified U.S. Patent Application.
3. I am familiar with the U.S. Patent & Trademark Office Action dated July 29, 2005 on the above identified application, in which the Examiner cited Donohue et al, U.S. Patent Application 2004/0266717 as part of a rejection of the claimed invention therein under 35 U.S.C. 103.
4. That the effective filing date of the Donohue et al reference is the date of the priority Provisional patent application Serial No. 60/230,311, filed September 6, 2000.
5. That the aforementioned reference to Donohue et al is not prior art to the above-identified application, inasmuch as the invention disclosed and claimed in the above-identified patent application of Feld et al was

reduced to practice, and thus made, prior to the effective September 6, 2000 filing date of Donohue et al.

6. In evidence thereof, I attach herewith
    a. A copy of a Ph.D. research proposal (in Hebrew), Exhibit A, of Yair Feld submitted to me with a view to prepare a patent application thereon prior to September 6, 2000. I read, reviewed and understood the invention disclosed therein.
    b. A translation of the said Ph.D. research proposal into the English language, (Exhibit C). Figure 4, Paragraph 2 of the Preliminary Results Section of said Ph.D. research proposal, shows that the above identified disclosed and claimed invention was reduced to practice prior to September 6, 2000. Said English translation was prepared by me, and I am thoroughly conversant with both the Hebrew and English languages.
    c. I was instructed by Dr. Shmuel Liran, the authorized official of the Technion, in an email dated prior to September 6, 2000 (Exhibit D, date blackened out) to prepare and file a patent application according to the disclosure received from the inventors, which consisted of said Ph.D. research proposal.
7. That the rejection of claims of the Feld et al invention over any rejection that employs Donohue et al should be withdrawn since Donohue et al is not prior art relative to the invention that is the subject of the above-identified patent application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,



Gal Ehrlich

Date: October 18, 2005

EXHIBIT A

הערכה ארוכת טווח של תרביות תאי לב מעורבות

רקע מדעי

מערכת ההולכה החשמלית הנה מערכת מורכבת המשחקת תפקיד חשוב בפיזיולוגיה של הלב ובפתוגנזה של יצירת הפרעות קצב. הפרעות אלו מהוות גורם משמעותי לתחלואה ותמותה בעולם. כך לדוגמא טכיקרדיה חדרית מהווה גורם מרכזי למוות פתאומי, וגובה כ- 1200 איש ביום בארה"ב. בבסיס רוב הפרעות הקצב, ניתן למצוא הטרוגניות. הטרוגניות זו עשויה להיות חשמלית או סטרוקטורלית. באופן מסורתי, הטיפול בהפרעות קצב משלב פרמקולוגיה, קוצב מושתל, דפיברילטור ופרוצדורת צריבה תוך שימוש בקטטר, רובם ככולם מאפננים את הרכיב האלקטרופיזיולוגי אשר גורם להפרעת הקצב בלב. אחד החסרונות של טיפול תרופתי שהשפעתו אינה ספציפית לאזור יצירת ההפרעה, ולכן קשור בתופעות לוואי מרובות. גישה חדשנית אפשרית להבנת המנגנוניים הקשורים ביצירת ובטיפול בהפרעות קצב, אותה נבחן בעבודה זו, הנה הערכת הקשר בין התכונות החשמליות והמבניות של רקמת הלב ושינוי המצע האלקטרופיזיולוגי הפתולוגי ע"י התערבות מקומית באמצעות החדרת אלמנטים אקסיטביליים חדשים.

הולכה חשמלית בלב

המיוקרד מורכב מתאים בודדים, והתקדמות ההפעלה החשמלית מתווכת ע"י gap junctions, המקשרים בין הציטופלסמה של התאים. למרות המבנה הארכיטקטוני הבודד של התאים, שריר הלב נחשב כמימד רציף מבחינה חשמלית וכימית. הוכחות ניסיוניות להומוגניות הפונקציונלית נצפו בסיבי פורקניה וטרבקולר חדרי (Wiedmann, 1970). ניסיונות אלו הראו שההתפשטות האלקטרוטונית של הזרם מתפזרת על מספר רב של תאים, וניתנת לתיאור ע"י תיאורית הכבל. נוסף, התלות של מהירות ההולכה ב- dV/dt_{max} של פוטנציאל הפעולה, הראתה קשר ריבועי כפי שנצפה מתיאוריה זו.

מחקרים מאוחרים יותר דווחו על תוצאות סותרות לרציפות של תיאוריית הכבל. כך נמצא ש- dV/dt_{max} גדול יותר בהולכה לרוחב הסיבים לעומת הולכה אורכית (Spach et al, 1981). טיפול בחומרים מפרי צימוד כמו Heptanol ו- Octanol יצר התנהגות מורכבת שאינה מונוטונית, שלא ניתן להסבירה ע"י מדיום רציף חשמלית. בעקבות תוצאות אלו הוצע שאי רציפות מיקרוסקופית הקשורה ל- gap junction היא הגורם להתנהגות ההולכה בצורה שונה מתיאוריית הכבל. אי רציפויות אלה בהולכה יתכן וגורמות להולכה לא הומוגנית והתפתחות חסמי הולכה המהווים מצע להתפתחות להפרעות קצב. נוכחות אלמנטים שאינם תאי לב והפרעות בצימוד חשמלי בין התאים עלולים לגרום לשינויים בהולכה החשמלית ולהוות מצע להתפתחות הפרעות קצב.

הערכת התכונות הפונקציונאליות והמבניות של תרביות תאים

השיטות השונות לחקר הלב מתמקדות בעיקר בבחינה מאקרוסקופית של שריר לב (הלב השלם, שריר פפילרי וכו') או ברמת תא בודד (patch clamp). שיטות אלו לרוב מוגבלות לבחינת פרמטר אחד כגון: רישום חשמלי, מדדים מורפולוגיים, חדמיית סידן וכו' ואינן מאפשרות הערכה ארוכת טווח סימולטנית של מספר פרמטרים.

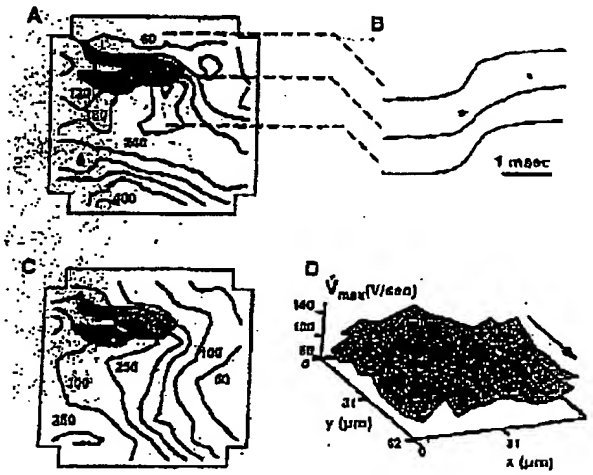
מודל in vitro מעניין להערכת תפקוד הלב הנו תרבית ראשונית חד-שכבתית של תאי לב שמקורה מלבבות של ולדות חולדה. תרביות אלו מורכבות ממשטח צפוף של מיוציטים הגדלים על מטריקס של קולגן ושומרים על תכונות הדומות ללב השלם. כך לדוגמה, מיוציטים בודדים בתרבית שומרים על צורת כישור, עם פיזור אחיד של connexin43 על הממברנה (Cabo et al, 1994). ניסיונות לחקור את התכונות החשמליות במודל זה התמקדו בשיטות שכללו בעיקר טכניקות להערכת התקדמות גל ההפעלה החשמלי ע"י מציאת זמן הפעלה מקומי באמצעות מדידת פוטנציאל תוך ותאי ע"י אלקטרודות חשמליות או ע"י שימוש בסמנים תלויי מתח (voltage sensitive dye). בעבודה זו בכוונתנו לקחת את המודל הזה, צעד אחד קדימה, ולבחון את תכונותיו הפיזיולוגיות, הפתולוגיות, והשפעת התערבויות מורכבות באמצעות שיטת מיפוי חדשנית. שיטה ייחודית זו, שאומצה מתחום הנוירופיזיולוגיה, משתמשת במערך רב-אלקטרודות על מנת להעריך לאורך זמן (20-5 יום) וברזולוציה מרחבית וזמנית גבוהה ביותר (100 µm, 25KHz) את התכונות החשמליות של רקמות אקסיטביליות. מערכת זו מאפשרת לראשונה לקשר בין התכונות הפונקציונליות (פעילות חשמלית ומכנית) והמבניות (באמצעות שיטות אימונוהיסטוכימיות) של הרקמה המיקרוסקופית לאורך זמן. יתר על כן האפשרות לקשר בין המבנה והתפקוד המיקרוסקופי של הרקמה, מהווה מערכת מודל אידיאלית להערכת תרביות תאים מעורבות.

קשר בין פיברובלסטים לקרדיומיוציטים

תרביות תאי לב מוולדות של חולדות מכילות בעיקר פיברובלסטים וקרדיומיוציטים. רוב התאים הם מיוציטים המצומדים חשמלית דרך gap junction ומתכווצים באופן סינכרוני. פיברובלסטים בלב, מקושרים חשמלית אחד לשני ולמיוציטים עם מוליכות בין תאית נמוכה. למרות זאת, נצפתה התכווצות סינכרונית בין מיוציטים המקושרים בינהם דרך פיברובלסט. תצפית זו הוכיחה קיום מעבר גרוי חשמלי באופן פסיבי דרך תאים שאינם אקסיטביליים בלב (Rook et al, 1992). בצמדי פיברובלסטים וצמדים של פיברובלסט-מיוציט, לא נמצאה רגישות פנימית למתח בינהם כאשר ההולכה הייתה מעל 2-1 nS, לעומת זאת, כאשר ההולכת הייתה נמוכה מ- 1 nS, נמצא gating תלוי במתח. הסתברות להימצאות במצב פתוח של gap junction ירדה ככל שעלה המתח על הצומת. למרות זאת, נשמרה הולכה שארית שאינה תלויה במתח. המוליכות דרך gap junction בודד בין פיברובלסטים נמדדה 21 pS ובין צמדים הטרולוגיים מיוציט-פיברובלסט 32 pS לעומת שני מיוציטים gap junction עיקרי 43 pS. בצביעות היסטוכימיות עם נוגדן ל- connexin43 נראתה צביעה בין מיוציטים ובין מיוציטים לפיברובלסטים, אך לא נראתה צביעה בין פיברובלסטים (Rook et al, 1992).

השפעת פיברובלסטים על הולכה

תאים שאינם מיוציטים בתרבית (פיברובלסטים ותאים אפיתליואידים) עשויים להיות קשורים אלקטרוטונית (Rook et al,1992). כיוון שתאים אלה אינם אקסיטביליים הם מהווים מקור חשוב של current sink, ולפיכך מורידים את dV/dt_{max} במיוציטים מסביבם, ובכך גורמים לעליה בסיכויים לחסימת הולכה ולהופעת הפרעות קצב.



איור 1 : השפעות תאים שאינם מיוציטים על הולכת הפולס החשמלי. ב- A ניתן לראות מפת אקטיבציה בגוון רוחבי כאשר הפיברובלסטים צבועים בשחור, וב- C ההולכת תיא אורכית. ב- B ניתן לראות את ה- upstroke של פוטנציאל פעולה בשני מיוציטים ובאמצע בפיברובלסט. ב- D ניתן לראות ממה טופוגרפית של - dV/dt_{max} ניתן לראות בבירור את השקע באזור הפיברובלסטים והמיוציטים שמסביבם. (Fast et al, 1996)

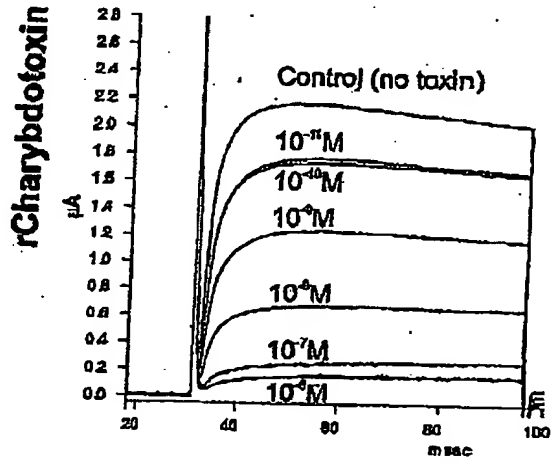
הרישום החשמלי מתאים פיברובלסטים בזמן מעבר הגירוי החשמלי מעיד על הימצאות gap junction בין המיוציטים לפיברובלסטים. הסטייה של חזית הגל כתוצאה מהימצאות פיברובלסטים כפי שמודגם באיור 1, מאשרת את הממצא הנייל. במהלך התקדמות הגל, הפיברובלסטים "שואבים" זרם מהמיוציטים, ובכך dV/dt_{max} ומהירות ההולכה במיוציטים באיזורים אלה יורדים. הרלוונטיות של ממצא זה באיהומוגניות בהולכה in vivo אינה ברורה. לא ברור אם מיוציטים יוצרים gap junction עם פיברובלסטים in vivo באותה המידה כמו בתרבית.

במידה וכן, ממצא זה יכול להיות משמעותי בפתוגנזה הקשורה בתהליכי החלמה מאוטם ביחוד בגבולותיו, בקרדיומיופטיה, ובהיפרטרופיה חדרית.

תעלות אשלגן Kv1.3

תעלות אשלגן Kv1.3 מתבטאות במוח, בלימפוציטים, וברקמות אחרות, אך לא בכמות משמעותית בלב. בנוסף התאפשר ביטוי ממברנלי של תעלה זו, באמצעות טרנספקציה במספר מערכות מודל תאיות (פיברובלסטים, Xenopus oocytes , תאי כלייה עובריים הומניים וכו'). בכוונתנו להשתמש בפיברובלסטים שעברו טרנספקציה עם תעלות אלו כחלק מתרביות מעורבות עם תאי לב כמודל אפשרי לשינוי המצע החשמלי של תאי הלב. לשימוש במערכת זו מספר יתרונות על מנת להעריך גישה טיפולית אפשרית חדשנית זו:

(1) ניתן לאפיין את התכונות החשמליות של תעלות אלו בצורה מפורטת (Marom et al, 1993).
(2) תאים אלו תוארו כיוצרים קשרים עם תאי לב.
(3) קיימת מערכת נוגדנים המאפשרת זיהוי של מיקום תאים אלו באמצעות צביעה אימונוהיסטוכימית ועל כן ניתן להעריך בנוסף להשפעתם הגלובלית גם את השפעתם המקומית.
(4) תעלה זו ניתנת לחסימה ספציפית ע"י Charybdotoxin – ראה איור 2.



איור 2 : השפעת Charybdotoxin על תעלות אשלגן Kv1.3
(התיסוי בוצע במעבדתו של פרופי שמעון פרום ע"י דייר ולדימיר ליאחוב).

מטרות המחקר

הערכת השפעתם של פיברובלסטים NIH 3T3 ופיברובלסטים NIH 3T3 שעברו הנדסה גנטית לביטוי תעלות יוניות על המצע האלקטרופיזיולוגי של תרביות תאי לב מעורבות לאורך זמן.

מטרות ספציפיות

1. התאמת מערכת רישום חשמלי חוץ תאי ברזולוציה מרחבית וזמנית גבוהה, ויצירת מודל ביולוגי הדיר.
2. אפיון חשמלי של מערכת הניסוי: הערכת התכונות האלקטרופיזיולוגיות של תרבית לאורך זמן. תוצאות אלה חשובות לשם הוכחת הדירות המערכת, וכבסיס להשוואה של השפעות התערבויות מורכבות על המצע האלקטרופיזיולוגי במודל זה.
3. אפיון חשמלי של תרבית מעורבת של קרדיומיוציטים ופיברובלסטים רגילים. הערכה זו עשויה להיות חשובה מהסיבות הבאות: (1) הבנת השינויים במצע האלקטרופיזיולוגי של הרקמה המתרחשים בזמן שינויים מבניים כמו התפתחות רקמה צלקתית והזדקנות הידועים כקשורים באריתמוגניות. (2) כימות השינויים הנגרמים בעקבות הוספת הפיברובלסטים לתרבית מבחינה מבנית, חשמלית, קצב פעימות ספונטני וכו', יהוו בסיס להשוואה של השפעת פיברובלסטים אקסיטביליים.
4. אפיון חשמלי של תרבית מעורבת של קרדיומיוציטים ופיברובלסטים שעברו הנדסה גנטית לביטוי תעלות אשלגן מסוג Kv1.3. אפיון זה חשוב על מנת לבחון היתכנות גישה זו. בפרט, נבחן השפעה אפשרית של תאים אלו על קצב פעימות הספונטני של התרבית, מהירות וצורת

ההולכה החשמלית הכללית והמקומית, תקופות רפרקטוריות ותכונות קלט פלט בתדרי קיצוב שונים.

5. הערכת תבניות זריעת פיברובלסטים שונות על תכונות התרבית.
6. הערכת השינוי האלקטרופיזיולגי בתרבית בעקבות הוספת פיברובלסטים עם תעלות נוספות.
7. אפיון ההשפעה האלקטרופיזיולוגית של הפיברובלסטים בתא מיוציט בודד. אפיון זה עשוי לאפשר הערכת המנגנון הפיזיולוגי הגורם לשינויים החשמליים והמבניים בתרבית בעקבות הוספת פיברובלסטים.
8. בדיקת יכולת דיכוי של אזורים בלב שעברו מודולציה לאקסיטבילית מוגברת. בדיקה זו תוכל לתת אינדיקציה לייתכנות גישה חדשנית זו.

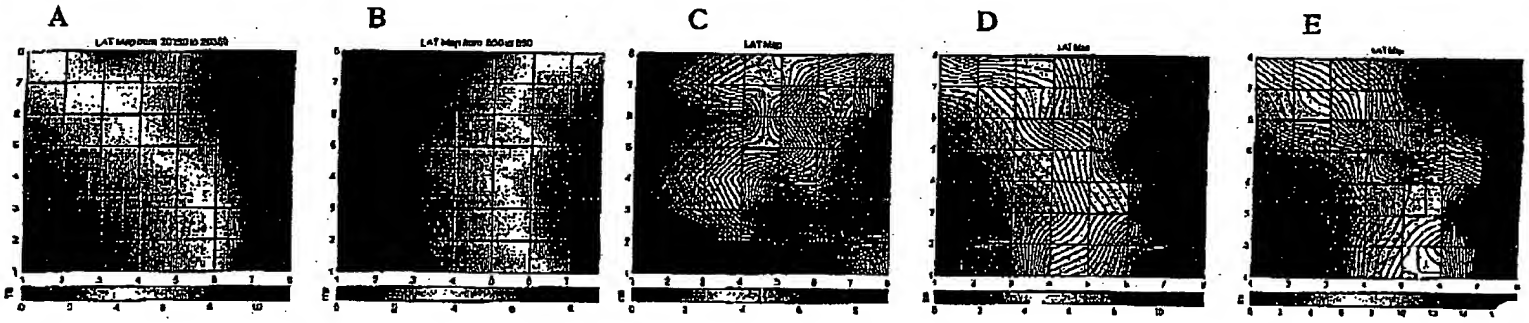
<u>תוכנית עבודה</u>

1. התאמת מערכת מיפוי חשמלית חוץ ותאית למדידות לאורך זמן בהתאם למערכת שפותחה במעבדתו של פרופ' עופר בינה ע"י גדעון מאירי ויותם רייזנר. הוקמה מערכת הכוללת מטריצה של 60 אלקטרודות המסוגלת לדגום בתדירות 25 KHz, האלקטרודות נמצאות במרחק של 0.1 או 0.2 mm אחת מהשניה (Igelmund et al, 1999). המערכת כוללת בקר טמפרטורה ומתחבר אליה בלון גז המכיל 95% אויר ו- 5% CO2 ע"מ לאפשר תנאי אינקובציה למדידות ארוכות טווח. עיבוד הנתונים נעשה ע"י תוכנה שפותחה במיוחד. התוכנה מייצרת מפת אקטיבציה כאשר זמן האקטיבציה נקבע ע"פ הגזרת המינימלית של הרישום החוץ תאי (Spach et al, 1981) dV/dt_{min}. מהירות ההולכה מחושבת מתוך מטריצה של זמן האקטיבציה.
2. אפיון אלקטרופיזיולוגי של תרבית קרדיומיוציטים לאורך זמן. מערכת המודל מבוססת על הפקת תרבית תאי לב מוולדות חולדה מסוג Sprague-Dawley בנות 2-1 ימים. מערכת זו של תרביות חד שכבתיות הינה מערכת מקובלת בספרות לחקר של רקמת הלב (Fast et al, 1996). התאים גדלים בצלחות גדול מיוחדות על גבי מטריצת אלקטרודות, ומוכנסות לאינקובציה ב- 37 5% CO_2 95% אויר. המדיום מוחלף כל יומיים. בכל יום תבוצע מדידה לאחר קיבוע תאים, לצורך הערכת השתנות התכונות האלקטרופיזיולוגית עם הזמן. הפרמטרים שיבדקו: תדירות ספונטנית, מהירות ומסלולי הולכה חשמלית ויציבות פרמטרים אלו לאורך זמן, ישמשו כבקורת להערכת שינויים אפשריים בשלביים הבאים של המחקר.
3. אפיון אלקטרופיזיולוגי של תרבית קרדיומיוציטים עם פיברובלסטים לאורך זמן. התרבית המעורבת תופק באותה השיטה ובין הימים ה- 7-3, יזרעו פיברובלסטים מסוג cell) NIH 3T3 line של פיברובלסטים מעכבר). בכל יום תבוצע מדידה והערכת הפרמטרים החשמליים המוזכרים ב-סעיף הקודם, וזאת על מנת: (1) להעריך את השפעת הפיברובלסטים על התכונות הגלובליות והמקומיות של הרקמה. (2) כימות השינויים הנגרמים בעקבות הוספת הפיברובלסטים לתרבית מבחינה מבנית, וחשמלי תהווה בסיס להשוואה של השפעת פיברובלסטים אקסיטביליים.
4. אפיון אלקטרופיזיולוגי של תרבית קרדיומיוציטים עם פיברובלסטים, שעברו הנדסה גנטית לביטוי תעלות אשלגן מסוג Kv1.3 (טרנספקציה של הפלסמיד לתעלת אשלגן מסוג Kv1.3 לפיברובלסט NIH 3T3 בוצעה במעבדתו של פרופ' שמעון מרום ע"י ד"ר מאירה פונקי, לאורך זמן. התרבית המעורבת תופק באותה השיטה ובין ימים 7-3, יזרעו פיברובלסטים NIH 3T3 (cell line של פיברובלסטים מעכבר) שעברו הנדסה גנטית בעזרת פלסמיד לביטוי תעלת אשלגן Kv1.3. תאי הפיברובלסטים כאשר הם גדלים בנפרד יעברו סלקציה ע"י אנטיביוטיקה. בכל יום תבוצע מדידה לאחר קיבוע תאים, לצורך הערכה אלקטרופיזיולוגית. במקביל תבוצע השוואה לתרביות ללא פיברובלסטים ותרבית עם פיברובלסטים ללא טרנספקציה, ותיבדק התגובה של התרביות השונות לחוסם ספציפי של תעלות אשלגן Kv1.3 - CTX. ספציפית, לאחר התייצבות המערכת והערכה ראשונית של פרמטרים החשמליים הנבדקים, יוסף CTX בריכוזים עולים של 0.1 nM, 1 nM, 10 nM, 100 nM. והתכונות של המערכת תיבדקנה בכל אחד מהשלבים. באופן דומה תתבצע הערכת השפעת CTX גם על תרבית הבקרה ותרביות הפיברובלסטים הרגילות.
5. אפיון השפעת הוספת הפיברובלסטים על תכונות הרפרקטוריות של הרקמה. בנוסף להשפעת אלמנטים אקסיטבילים אלו על התכונות האקסיטובילית של הרקמה, יבחנו גם השפעות אפשריות על הרפולריזציה. השפעות אלו יבחנו ע"י שינויים מקומיים וגלובלים בתקופה הרפרקטורית האפקטיבית של הרקמה. שינויים אלו עשויים לנבוע מזרמים אלקטרוטוניים בין הקרדיומיוציטים והפיברובלסטים גם במהלך פוטנציאל הפעולה.
6. אפיון תכונות הקלט-פלט של הרקמה המעורבת תחת תבניות גירוי שונות.

7. אפיון השפעת תבניות זריעה שונות על התכונות האלקטרופיזיולוגיות של התרבית. בניסוי זה נבדוק את השפעת פיברובלסטים אלו על תכונות הרקמה בדומה למה שתואר בסעיפים הקודמים, רק שהזריעה אינה דיפוזית אקראית, אלא בתבניות מתוכננות.
8. הערכת השינוי האלקטרופיזיולוגי בתרבית בעקבות הוספת פיברובלסטים עם תעלות אחרות. באופן דומה נבדוק את השפעת אלמנטים אקסיטביליים אחרים (פיברובלסטים שעברו טרנספקציה לביטוי תעלות נתרן ופיברובלסטים שעברו טרנספקציה לביטוי תעלות נתרן ואשלגן) על המצע האלקטרופיזיולוגי.
9. אפיון ההשפעה האלקטרופיזיולוגית של הפיברובלסטים על תא מיוציט בודד. הפיברובלסטים יצבעו ע"י Fast dye O (חומר צבע ויטאלי ממברנלי) לפני הזריעה בתרבית המיוציטים. בניסוי patch clamp על תא מיוציט בסביבה של פיברובלסטים צבועים, נאפיין את אופי פוטנציאל הפעולה שלו, תחת ריכוזים עולים של CTX, לעומת מיוציט שלא נמצאים פיברובלסטים צבועים בסביבתו.
10. בדיקת התכונות של גישה זו בלב השלם (במדה ויתאפשר). בניסוי זה נבחן אפשרות השפעה של תאים אלו על התכונות האקסיטביליות של הלב השלם. נבחן שיטות הזרקה אפשריות, ונעריך את יכולת ההישרדות של תאים אלו ברקמת הלב השלם. על מנת לבחון השפעה אפשרית של גישה זו לשינוי התכונות החשמליות של הלב נזריק תאים אלו לאזור ה- AV node של חולדות (ע"פ מודל מקובל בספרות) ונבחן את תכונותיו לפני ואחרי ההשתלה ותחת מתן CTX. הפרמטרים שיבדקו יכללו : זמן ההולכה, תקופה רפרקטורית ותדר Wenkebach.

<u>תוצאות ראשוניות</u>

1. בוצעו מספר ניסויים ע"מ לאפיין את המערכת בתרביות תאי לב רגילות לאורך זמן. באיור 3 ניתן לראות מס' מפות אקטיבציה שנלקחו מאותה התרבית בימים שונים. ניתן לראות את הדימיון בין מפות האקטיבציה A, D ו- E, אשר ממחיש את הדירות המערכת. מהירות ההולכה בתרביות כפי שניתן לראות מטבלה 1 נעות בין [m/s] 0.1 ל- [m/s] 0.2, ערכים התואמים את ערכי מהירות ההולכה בתרביות חד שכבתיות המדווחים בספרות (Fast VG et al, 1997).

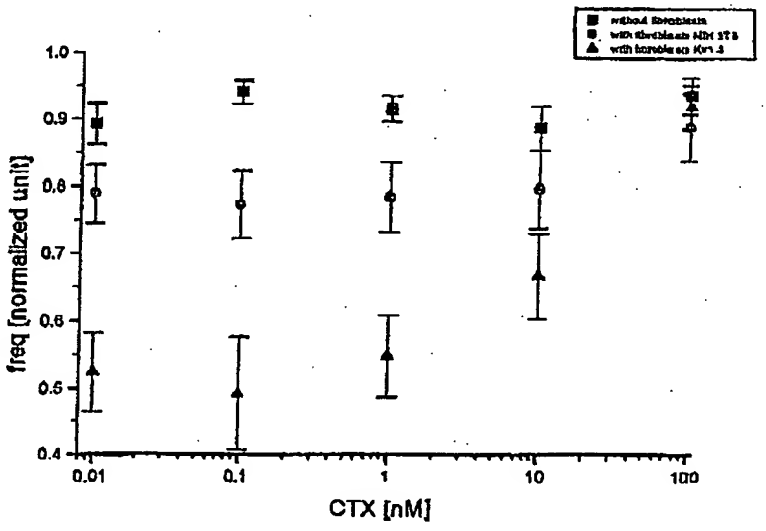


איור 3 : מוצגות חמש מפות אקטיבציה מתרבית רגילה, כאשר מפת A מחיום התחישי מחפקת התרבית, מפת B מחיום חשישי וכך הלאה. במפות D,C ו- E חוסמו קווים אינוכרוניים, תממחישים טוב יותר את צורת התפשטות הגירוי בתרבית.

day	conduction velocity; peripheral [m/s]	conduction velocity; average [m/s]	conduction velocity; standard deviation [m/s]
A	0.14736	0.18377	0.16458
B	0.189284	0.184229	0.05369
C	0.215641589	0.214247005	0.190768179
D	0.155662581	0.146432193	0.061536928
E	0.10262041	0.141289112	0.162845482

טבלה 1 : בטבלה זו מוצגות מהירויות החולכה המתושבות עבור כל אחת ממפות האקטיבציה באיור 3. מהירות החולכת חושבה בשתי שיטות : 1. לפי האלקטרוזות בחיקף (conduction velocity peripheral) . 2. בכל אלקטרודה חושבה מהירות החולכה לפי ארבע האלקטרודות הסמוכות לה, ולאחר מכן בוצע ממוצע של המהירויות בכל האלקטרודות (conduction velocity average). בנוסף, חושבה סטיית התקן של מהירות החולכה בכל האלקטרודות (conduction velocity standard deviation).

2. בוצע ניסוי כמתואר בסעיף 4 של תוכנית העבודה. קצב הפעימות הספונטני של התרביות נמדד בתרביות הבקרה ללא פיברובלסטים, בתרביות עם פיברובלסטים שעברו טרנספקציה לביטוי תעלות אשלגן ותרביות עם פיברובלסטים ללא טרנספקציה, בריכוזי CTX עולים. מהתבוננות באיור 4 ניתן לראות כי חלה עלייה של 42% בתדירות הפעימות לאחר הוספת CTX בתרבית עם פיברובלסטים שעברו טרנספקציה לביטוי תעלות אשלגן, לעומת עליה של 5% בלבד בתרבית ללא פיברובלסטים ו- 12% בתרביות עם פיברובלסטים ללא טרנספקציה. הסבר אפשרי לתופעה זו עשוי לנבוע מהיפרפולריזציה הנגרמת כתוצאה מפתיחת תעלות Kv1.3 בפיברובלסטים בתרבית, בזמן פוטנציאל פעולה, הגורמת להתארכות פאזה 4 בתאי הלב. בנוסף, יתכן שמוקדי פעילות מסויימים נמצאים תחת דיכוי של הפיברובלסטים, ולאחר הוספת CTX המוקדים הנייל משתחררים. בתרביות הבקרה השיניים בקצב הפעימות קטנים בהרבה ולא בהכרח קשורים לריכוז ה- CTX במדיום.



איור 4 : תנובות תרבית ללא פיברובלסטים, ותרבית עם פיברובלסטים ללא טרנספקציה (NIH 3T3), תרבית עם פיברובלסטים שעברו תנוסח גנטית לביטוי תעלות אשלגן Kv1.3, לריכוזים עולים של Charybdotoxin. n– מספר המדידות, c–מספר התרביות (witout fib - c=8;n=16, NIH 3T3 - c=3;n=27, Kv1.3 - c=4;n=26).

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EXHIBIT B

THE GRADUATE SCHOOL
TECHNION - ISRAEL INSTITUTE OF TECHNOLOGY

הטכניון - מכון טכנולוגי לישראל
בית הספר ללימודי מוסמכים

[redacted]

TO WHOM IT MAY CONCERN

This is to certify that Mr. **Yair Feld** (Student No. 028874485) has presented the enclosed **PhD** research proposal together with his request to join the direct **PhD** track, to the faculty of Medicine for approval on [redacted] with Prof. Lior Gepstein as his supervisor.

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שלבי גמר	מזכירות ואישורים	מעקב	מלגות	רישום	לשכת הדיקן
Graduation	Secretariat	Follow-up	Scholarships	Registration	Dean's Office
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EXHIBIT C

Long term evaluation of hybrid cardiomyocytic cultures

Scientific background

The electrical conduction system is a complex system that plays an important role in heart physiology and the pathogenesis of cardiac arrhythmias. These abnormalities are a significant cause of worldwide morbidity and mortality. For example: ventricular tachycardia is a major cause of sudden death, responsible for the lives of 1,200 people a day in the USA alone. The substrate for most of the arrhythmias is heterogeneity. Heterogeneity can be electrical or structural. Traditionally, the treatment combines medications, pacemaker, defibrillator and catheter based ablation procedure, all aims to modify the electrophysiological abnormality that causes the arrhythmia. One of the disadvantages of the pharmacological treatment is the non-specific affect to on the abnormal area, which is the reason for the many side effects.
A possible novel approach to understand the mechanisms that are associated with the generation and treatment of cardiac arrhythmias, which we will test in this work, is the evaluation of the electrical and structural properties of the cardiac tissue and modification of the pathological electrophysiological substrate by local intervention by introducing new excitable elements.

Electricle conduction in the heart

The myocardium is composed from discrete cells, and the electrical activation propagation is mediated through gap junctions, that connect the cytoplasm's of the cells. Even though the heart is a discrete architectonic structure of cells, the heart muscle is considered a continuous medium electrically and chemically. Experimental evidence of homogeneous functionality was observed in Purkinia fibers and ventricular trabeculae (Wiedman, 1970). These experiments demonstrated that the electrotonic currents spreads on a large number of cells, and can be described by the cable theory. Moreover, the dependency of the conduction in dV/dt_{max} of the action potential demonstrated a quadratic relation as predicted by this theory.
Later researches reported contradicting results to the continuity of the cable theory. For example, it was observed that dV/dt_{max} was larger during transverse conduction compared to longitudinal conduction (Spach et al, 1981). Application of uncouplers like Heptanol and Octanol produced complex behavior that is not monotonic, that cannot be explained by a continuous electrical medium. Therefore, it was suggested that microscopic discontinuity was related to gap junction is the cause of the deviation of the conduction behavior from the cable theory. These discontinuities in conduction may cause inhomogeneous conduction and the development of conduction blocks that serve as a substrate to the development of cardiac arrhythmias. The presence of elements that are not cardiac cells and abnormality in the coupling of the cells could cause changes in the electrical conduction and become a substrate for the development of cardiac arrhythmias.

Evaluation of the functional and structural properties of cell cultures

The different methods for cardiovascular research focus on macroscopic evaluation of the myocardium (whole heart, papillary muscle etc') or single cell (patch clamp). These methods are usually limited to one parameter analysis, for example: electrical recording, morphological parameters, calcium imaging etc' and do not enable simultaneous long term evaluation of a number of parameters.
An interesting in vitro model for cardiac function evaluation is primary monolayer cardiomyocytes culture originating from neonatal rat hearts. These culture are composed of dense myocyte layer growing on collagen matrix and express properties similar to the whole heart. For example, single myocytes in culture are spindle like, with a uniform expression of connexin43 on their membrane (Cabo et al, 1994). Experiment on this model focused on methods that include techniques for evaluation of the electrical propagation wave by recording the local activation time using electrical electrodes or voltage sensitive dyes. In this work, we want to take this model one-step forward and to test the physiologic and pathologic properties and the effect of complex intervention using a novel mapping system. This system, adapted from the neurophysiology field, uses an array of electrodes in order to evaluate long term (5-20 days) and at a high spatial and temporal resolution (100mm, 25KHz) the electrical properties of excitable tissues. This system enable for the first time association between the function (electrical and mechanical activity) and morphology (using immunohistochemistry methods) of the tissue at a microscopic level for long term. Moreover, the possibility to associate between the microscopic morphology and function of the tissue is an ideal model for evaluation of hybrid cell cultures.

Fibroblasts-cardiomyocytes coupling

Neonatal rat cardiomyocytic cultures are composed of mainly fibroblasts and cardiomyocytes. Most of the cells are myocytes electrically coupled through gap junction and contract synchronously. Fibroblasts in the heart are electrically coupled to one another and to cardiomyocytes with a low intercellular conductance. However, synchronous contraction of cardiomyocytes electrically coupled through a fibroblast was reported. This observation proved the passive transition of electrical stimulus through non-excitable cells in the heart (Rook et al, 1992). In couplets of fibroblasts and couplets of fibroblast-myocyte, no internal voltage sensitivity was observed when conductance was above 1-2 nS, however, when conductance was below 1 nS, a voltage gated property was recorded. The probability of gap junction to be in an open state reduced as the voltage on the junction increased. However, a residual conductivity non-voltage dependent was preserved. The conductance between a single gap junction between fibroblasts was recorded 21 pS and between heterogeneous couplets myocyte-fibroblast 32 pS verses two myocyte main gap junction 43 pS. In histochemistry positive staining with antibody to connexin43 was observed between myocytes and between myocytes and fibroblasts, but not between fibroblasts (Rook et al, 1992).

The effect of fibroblast on conduction

Non myocyte cells in culture (fibroblasts and epithelia cells) could be electrically coupled (Rook et al, 1992). Since these cells are not excitable they serve as an important source of current sink, and therefore, reduces dV/dt_{max} in the surrounding myocytes, and increases the chance for conduction block and the development of cardiac arrhythmias.

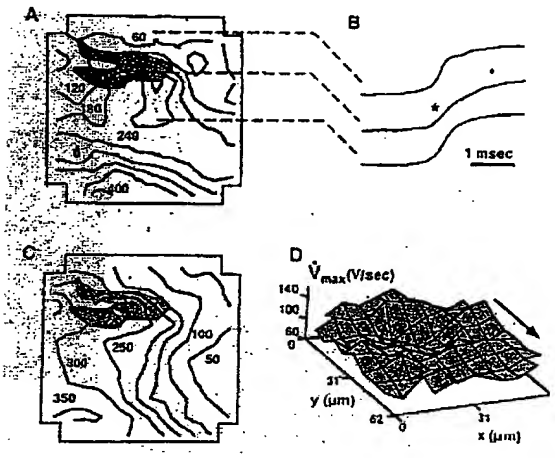

Figure 1: The effect of non-myocyte cells on the propagation wave. In A is an activation map in a transverse direction, were the fibroblast are labeled in black, and in C the propagation is longitudinal. In B the upstroke of the action potential in two myocytes and in the middle in a fibroblast. In D topographic map of dV/dt_{max} the sink in the middle is clearly visible in the area of the fibroblast and the surrounding myocytes. (Fast et al, 1996)

The electrical recording from fibroblast cells during the propagation wave indicates the existence of gap junction between the myocytes and the fibroblasts. The deviation of the propagation wave due to the fibroblast as demonstrated in figure 1, confirms this finding. During the propagation of the activation wave, the fibroblasts "suck" current from the myocytes, thus reducing dV/dt_{max} and conduction velocity in these areas. The relevance of this finding to heterogeneity in conduction in vivo is not clear. It is not clear whether myocytes form gap junctions with fibroblasts in vivo as well as in culture. If they do, this finding can be significant in the pathogenesis related to recovery from infarct specifically in the borders, in cardiomyopathy, and ventricular hypertrophy.

Kv1.3 potassium channels

Kv1.3 potassium channels are expressed in the brain, lymphocytes, and other tissues, but not significantly in the heart. Moreover, membrane expression of this channel was successful in a number of cellular model (fibroblasts, Xenopus oocytes, human

embryonic kidney cells etc'). We intend to use fibroblasts transfected with these channels as part of hybrid culture with cardiac cells as a possible model for modifying the electrophysiological substrate of the cardiomyocytes. Using this system has a number of possible advantages for testing this novel treatment approach:

(1) It is possible to characterize the electrophysiological properties of this channel in details (Marom et al, 1993).
(2) It was demonstrated that these cells coupled with cardiomyocytes.
(3) There is a set of antibodies that enable the detection of the cells using immunohistochemistry that enable evaluation of the cells local affect as well as global effect.
(4) This channel can be block specifically by Charybdotoxin – see figure 2.

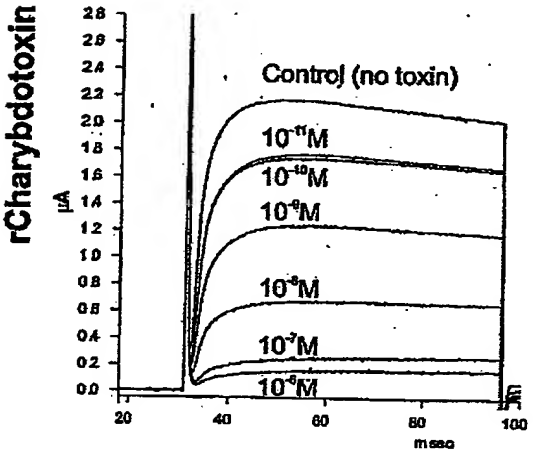

Figure 2: The effect of Charybdotoxin on Kv1.3 potassium channels (Alomone).

Research aims

Development of experimental system model that will enable long term evaluation of the effect of external excitable elements on the electrophysiological substrate of cardiomyocytic cultures.

Specific aims

1. Establishment of electrical extra cellular signal recording system with a high spatial and temporal resolution, and generation of a reproducible biologic model.
2. Electrical characterization of the experimental system: long term evaluation of the electrophysiological properties of cultures. These results are important in order to demonstrate the reproducibility of the system, and as a control for the effect of complex intervention on the electrophysiological substrate in this model.
3. Electrical characterization of hybrid cultures of cardiomyocytes and unmodified fibroblasts. This evaluation is important due to the following reasons: (1) understanding the changes in the electrophysiology of the tissue that occur during structural changes like generation of scar tissue and aging that are known to be

associated with arrhythmogenisity. (2) quantifying the changes due to the seeding of the fibroblasts to the cultures in terms of structure, electrical, spontaneous beating rate etc', will serve as baseline for comparison of the effect of excitable fibroblasts.

4. Electrical characterization of hybrid cultures of cardiomyocytes and genetically modified fibroblasts expressing the Kv1.3 potassium channel. . This characterization is important in order to test the feasibility of this approach. In particular, we will test the possible effect of these cells on the spontaneous beating rate of the culture, velocity and form of conduction overall and local, refractory period and input output properties at different stimulus rates.
5. Evaluation of different seeding patterns on the culture properties.
6. Evaluation of the electrophysiological changes in culture seeded with fibroblast expressing other ion channels.
7. Characterization of the effect of the fibroblasts on a single myocyte. This characterization may enable evaluation of the physiological mechanism that responsible for the electrical and structural changes in the culture due to the fibroblasts seeding.
8. Evaluation of the possibility to suppress areas in the heart that were modify to hyper-excitability. This test will give indication to the feasibility of this novel approach.

Working plan

1. Establishment of a long term mapping system for electrical extra-cellular signal recordings. A system was established with an array of 60 electrodes capable of recording at a 25 KHz, the electrodes are 0.1 or 0.2 mm apart (Igelmund et al, 1999). The system includes a temperature controller, and connected to a supply of 95% air and 5% CO_2 in order to enable incubation conditions for long-term recordings. Data analysis is performed using custom made program specially designed for this project. The program generate an activation map were the activation time is set as the minimal derivative of the extra-cellular signal dV/dt_{min} (Spach et al, 1981). The conduction velocity is calculated from the matrix of the activation times.
2. Long-term electrophysiological characterization of cardiomyocytic culture. The system model is based on production of Sprague-Dawley neonatal rat cardiomyocytes 1-2 days old. This system of monolayer culture is well known in the literature for cardiac tissue research (Fast et al, 1996). The cells are plated on a special dish with a matrix of electrode, and are put in incubation at 37°, 5% CO_2 95% air. The medium is replaced every second day. Each day following condition stabilizing a recording will be taken in order to evaluate the changes in the electrophysiological properties with time. The parameters that will be evaluated are: spontaneous rate, velocity and tract of conduction and the stability of these parameters over time, will serve as control to the evaluation of the possible changes in the next steps of the research.
3. Long-term electrophysiological characterization of cardiomyocytic culture with fibroblasts. The hybrid culture will be produced using the same method and

during days 3-7, fibroblasts NIH 3T3 (fibroblasts cell line from mouse) will be addaed to the culture. Each day recordings and evaluation of the electrical parameters mentioned in the previous section will be conducted in order to: (1) evaluate the effect of the fibroblast globally and locally on the tissue. (2) quantify the changes that are caused by the adding of the fibroblasts to the culture morphologically, and electrically will serve as baseline for comparison to the effect of excitable fibroblasts.

4. Long-term electrophysiological characterization of cardiomyocytic culture, with fibroblasts genetically modified to express the Kv1.3 potassium channel. The hybrid culture will be produced using the same method and during days 3-7, fibroblasts NIH 3T3 (fibroblasts cell line from mouse) that were genetically engineered by a plasmid to express Kv1.3 potassium channel. The fibroblasts when grown separately will be under selection by antibiotics. Each day following condition stabilization a recording will be performed for electrophysiological evaluation. In parallel a comparison to culture without fibroblasts and cultures with unmodified fibroblasts will be performed, and the respond of the different cultures to CTX – a specific Kv1.3 potassium channel blocker will be evaluated. Specifically, following stabilization of the system and primary evaluation of the electrical parameters tested, CTX at growing concentrations of 0.1nM, 1nM, 10nM, 100nM will be administered, and the properties of the system will checked at each stage. Similarly, the effect of CTX will be evaluated on control cultures and the cultures with unmodified fibroblasts.
5. Characterization of the effect of adding fibroblasts on the refractory period of the tissue. Moreover, the effect of adding these excitable elements on the excitability properties of the tissue, and possible effects on repolarization. Thes effect will be evaluated by local and global changes in the effective refractory period of the tissue. Thes changes may occur due to electrotonic currents between cardiomyocytes and fibroblasts also during the action potential.
6. Characterization of the input-output properties of the hybrid cultures under different stimulus patterns.
7. Characterization of the effect of different seeding patterns on the electrophysiological properties of the cultures. In this experiment we will test the effect of these fibroblasts on the tissue properties similarly to what was described in the previous sections, only the seeding pattern will not be randomly and diffuse, but in a predetermined patterns.
8. Evaluation of the electrophysiological changes in cultures due to adding fibroblasts with different ion channels. Similarly we will test the effect of other excitable elements (fibroblasts transfected with sodium channels and fibroblasts co-transfected with sodium and potassium channels) on the electrophysiological substrate.
9. Characterization of the effect of the fibroblasts on a single myocyte. The fibroblasts will be labeled with a vital dye before seeding in the cardiomyocyte culture. In a patch clamp experiment on a single myocyte in an area of labeled fibroblasts, we will characterize the nature of its action potential under increasing CTX concentrations, compared with myocytes that don't have labeled fibroblasts surrounding them.

10. Evaluation of this approach on the whole heart (if possible). In this experiment we will test the effect of these cells on the excitable properties of the intact heart. We will test different injection possibilities, and evaluate the potential; survival of the cells in the intact heart. In order to test the possible effect of this method on the electrical properties of the heart we will inject the cells to the area of the AV node (according to an acceptable model in the literature) and test its properties before and after transplantation and following CTX administration. The parameters that will be tested includes: conduction time, refractory period and Wenkebach frequency.

Preliminary results

1. A number of experiments were performed in order to characterize long-term the system on regular cultured cardiomyocytes. In figure 3 are seen a number of activation map recorded from the same culture in different days. The similarity of maps A, D and E is obvious and indicate the reproducibility of the system. The conduction velocity in the cultures as can be seen in table 1 are in the range of 0.1 [m/s] – 0.2 [m/s], which are similar to the conduction velocity in monolayer cultures reported in the literature (Fast VG et al, 1997).

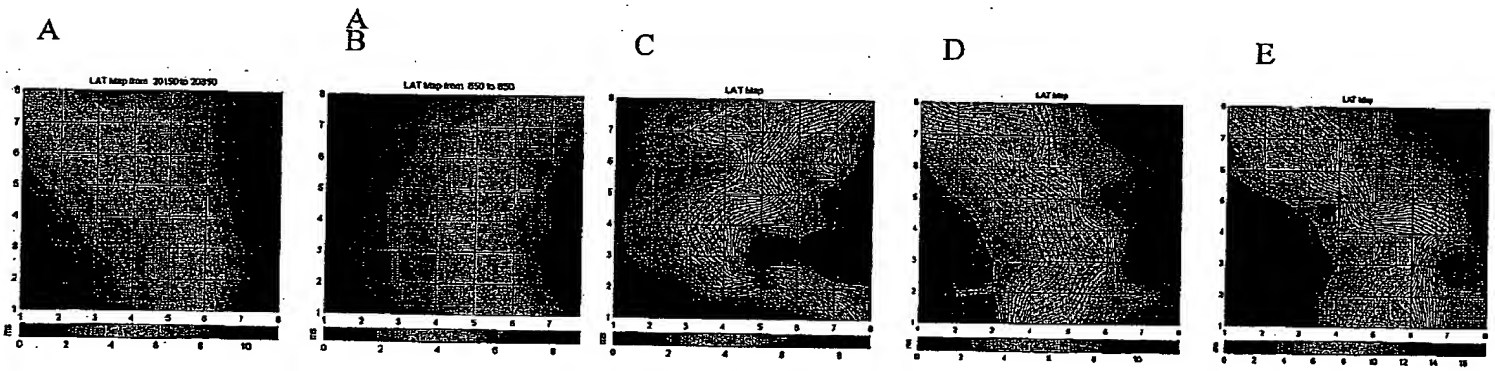

Figure 3: Presented are five activation maps from regular culture, where map A from the fifth day from culture production, map B from the sixth day and so on. In maps C, D, and E isochronous line were added, that demonstrate better the pattern of the propagation of the stimulus in the culture.

day	conduction velocity; peripheral [m/s]	conduction velocity; average [m/s]	conduction velocity; standard deviation [m/s]
A	0.14736	0.18377	0.16458
B	0.189284	0.184229	0.06369
C	0.215641589	0.214247005	0.190768179
D	0.155662581	0.146432193	0.061536928
E	0.10262041	0.141289112	0.162845482

Table 1: In the following table are the calculated conduction velocities for each of the activation maps in figure 3. The conduction velocity was calculated in two different methods: 1. according to the electrode in the periphery (conduction velocity peripheral). 2. In each electrode the conduction velocity was calculated according to her 4 adjacent electrodes, followed by averaging of the velocities in all electrodes (conduction velocity average). Moreover, the standard deviation of the conduction velocity of all electrodes was calculated (conduction velocity standard deviation).

2. The experiment described in section 4 of the working plan was conducted. The spontaneous beating rate of the cultures was recorded in control cultures without fibroblasts, in culture with transfected fibroblasts expressing potassium channels and culture with fibroblasts without transfection, in an increasing concentration of CTX. Looking at figure 4 it is possible to see a 42% increase in the beating rate following CTX administration in the cultures with fibroblasts that were transfected to express potassium channel, verses 5% increase only in the cultures without fibroblasts, and 12% increase in the cultures with fibroblasts without transfection. Possible explanation to the phenomena could be the hyperpolarization caused by the opening of the Kv1.3 channels in the fibroblasts in the culture, during the action potential, that causes an increase in phase 4 in the cardiac cells. Moreover, it is possible that activity areas are under suppression by the fibroblasts, and following CTX administration this focuses are released. In the control cultures the changes in the beating rate are much smaller and not necessarily related to the CTX concentration in the medium.

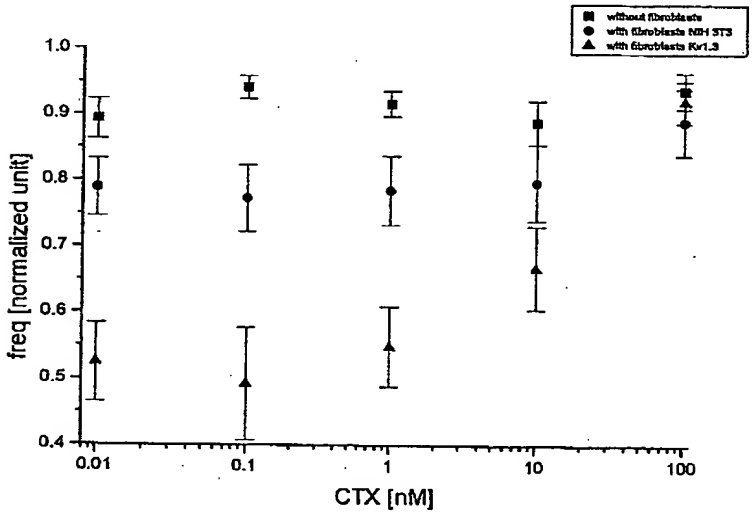

Figure 4: Respond of culture without transfection, culture with fibroblasts without transfection (NIH 3T3), and culture with fibroblasts genetically modified to express the Kv1.3 potassium channel, to an increasing concentration of Charybdotoxin. n – Number of measurements, c – number of cultures (witout fib - c=8;n=16, NIH 3T3 - c=3;n=27, Kv1.3 - c=4;n=26).

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18. Wiedmann S. Electrical constants of trabecular muscle from mammalian heart. J Physiol (Lond). 1970;210:1041-1054.

EXHIBIT 00/20989 D

Dr. Gal Ehrlich

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Cc: Tania (Ehrlich & Partners) (E-mail) <tania@ipatent.co.il>; Lowenstein Ami (E-mail) <lamiel@techunix.technion.ac.il>; Feld Yair (E-mail) <feld@techunix.technion.ac.il>
Sent: Tuesday, [redacted] 9:56 PM
Attach: 581 Inventors.doc
Subject: Drafting & filing a new US Patent application (pat file 581-US)

Dear Gal,

We herewith authorize you to draft and file a US patent application (*), according to the disclosure you received from the inventors.

Subject: Genetically engineered cellular grafts for the modulation and treatment of excitable tissues

Inventors: Yair Feld, Lior Gepstein, Shimon Marom, Meira Frank

Our ref: 581-US

Your fee for drafting the patent application (in addition to the filing costs) will be [redacted].

Attached is some data about the inventors.

Regards,

Shmuel

(*) According to the decision of Patent Committee 07-2000

TO YAIR FELD

Please fill in the missing addresses of the inventors (Dr. Lior Gepstein and Prof. Shimon Marom)
in the attached file (581 Inventors.doc)

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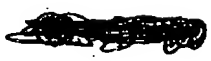

Modification of Cellular Communication by Gene Transfer

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Institute of Molecular Cardiobiology and Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

ABSTRACT: **Hope has been expressed that gene and cell therapies will one day reduce the morbidity and mortality associated with cardiovascular diseases. Work in these fields has shown that the road from bench to bedside is filled with obstacles. Still, the possibility for treatment or even cure of cardiac disease is real. Continuing work will improve understanding of the underlying physiology and vector biology. The current review focuses on the potential use of gene therapy to affect cellular communication. Included is a review of communication effects on a transcellular level with angiogenesis, AV nodal conduction and sinus nodal automaticity, and effects on an intracellular level with cardiac myocyte repolarization. Challenges facing the field of gene therapy are also reviewed. If these problems can be solved, gene therapy will become a viable alternative for clinical use.**

KEYWORDS: **angiogenesis; arrhythmia; gene therapy; growth factor; ion channel**

INTRODUCTION

Recent clinical trials have shown the utility of a variety of drugs and interventions for prevention and treatment of cardiac diseases, including antilipid therapy, adrenergic modulation, afterload reduction, antiplatelet agents, drug-eluting stents, implantable cardioverter-defibrillators, etc. Still, in spite of these innovations, the inevitable course for most patients is one of progressive disease. In part, this is due to the palliative nature of the available therapies. These limitations in currently available therapies have led to considerable speculation on the potential utility for gene or stem cell therapeutics for treatment of cardiovascular diseases. In the current review, we will focus on gene therapy as a means to alter intracellular or transcellular communication and discuss the use of genetic agents to achieve angiogenesis, to alter cardiac automaticity, and to modify conduction and repolarization. We will conclude with a discussion of the current problems preventing large-scale use of these therapies and potential answers to these problems.

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doi: 10.1196/annals.1341.014

INDUCTION OF ANGIOGENESIS IN ISCHEMIC REGIONS

The first cardiovascular gene therapy clinical trials focused on the use of growth factors to induce angiogenesis as a treatment for coronary disease. The underlying hypothesis was that focal application of gene transfer vectors encoding growth factors would lead to localized blood vessel formation, increasing the network of collateral vessels from less-diseased coronary vessels to areas served by partially or completely occluded coronary arteries. Animal work centered on use of two models: limb ischemia, caused by abrupt ligation of the femoral artery, and gradual coronary occlusion days or weeks after implantation of ameroid constrictors around coronary arteries. Both models employed otherwise healthy, young animals. Numerous growth factors were tested in these models, but the majority of the work to date has centered on use of either vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF). The VEGF work utilized two splice variants of the factor: $VEGF_{121}$ tested with adenoviral vectors, and $VEGF_{165}$ evaluated with plasmid vectors. All FGF testing has been performed with adenoviral vectors.

Animal work in both limb and coronary ischemia models gave suggestive evidence that gene therapy with any of these vectors was capable of inducing sufficient angiogenesis to relieve the ischemic burden.[1,2] Endpoints of these studies included microscopic quantification of blood vessel number in treated and control groups, various analyses of regional blood flow, and indirect indicators of ischemia and organ function. All endpoints were positive, providing evidence for initiation of several phase 1 clinical trials for limb and coronary ischemia.

The human work has been less convincing. Phase 1 trials for both peripheral and coronary ischemia showed improvement. The peripheral vascular disease trials documented marginal increases in the ankle-brachial index (0.33 improving to 0.48) but improved healing of ischemic ulcers.[3,4] Coronary phase 1 trials documented reductions in nitroglycerin usage and improved flow in dobutamine or treadmill stress tests.[5,6] Problems with these trials included the absence of blinding or of controls. Phase 2 trials included both control populations and investigator blinding to the treatment regimen. A peripheral phase 2 clinical trial using Ad-$VEGF_{121}$ found no change in the primary endpoint of peak walk time or the composite endpoint of peak walk time, ankle-brachial index, and time to onset of claudication.[7] The phase 2 coronary trial using Ad-$VEGF_{121}$ found no significant change in functional class, work ability, or nitrate use when comparing between control and treatment groups.[8] The Ad-FGF4 clinical found a nonsignificant trend toward improvement in perfusion.[9] Encouraging news from all trials is that the side effect profile appears to be minimal for all agents tested. To date, however, it is fair to say that angiogenesis gene therapy has been a disappointment. Ongoing studies will evaluate the use of multiple splice variants of VEGF or the hypoxia inducibility factor 1α to test the idea that more generalized activation of ischemia-reactive pathways might give better results than those seen with application of specific single agents like individual VEGF splice variants or the single serotype of FGF.

RECREATION OR AMPLIFICATION OF CARDIAC PACEMAKER ACTIVITY

Efforts to improve or recreate the sinus node have centered around three genes: the β-adrenergic receptor (βAR), the hyperpolarization-activated, cyclic nucleotide-

gated cation channel (HCN) gene encoding the I_f current, and the Kir 2.1 gene encoding the I_{K1} current.[10–12] The first reported manipulation of cardiac pacemaker activity involved amplification of the β-AR in the sinus node. Edelberg and co-workers mapped the earliest site of atrial activation, presumed to be the sinus node, and injected plasmid vectors containing the β-AR.[10] Two days after injection, the heart rate in the treatment group increased from a baseline of 108 beats per minute to a peak effect of 163 beats per minute. This effect was significantly reduced by day 3 and eliminated by day 4. Immunostaining results showed the presence of β-AR only in the sinus nodes of the treatment group. Comparing treatment to control groups showed a significant difference in heart rate, but no quantitation of gene transfer or comparison of expression level to effect was documented. Given the usual time course of gene expression after most gene transfer techniques, where peak effects occur 5 to 7 days after plasmid or adenovirus injection and gene expression is eliminated 2 to 3 weeks after injection, the results of these studies are difficult to explain and further investigation is warranted.

Subsequent to the initial report of amplified sinus node activity with β-AR gene transfer, Miake and colleagues showed that gene transfer of a dominant negative mutant of Kir 2.1 (GYG144-146AAA) caused automaticity in normally quiescent ventricular cells.[12] Kir 2.1 is the major subunit of the inward rectifier current I_{K1}, which is responsible for maintaining cellular resting membrane potential. In a guinea pig model, Miake *et al.* delivered adenoviruses throughout the ventricles using an aortic cross-clamping method initially reported by Hajjar *et al.*[13] They documented gene transfer to 20% of cells using the green fluorescent protein (GFP) reporter, and with the Kir 2.1 AAA dominant negative mutant they found increased automaticity on both a cellular and whole heart level (FIG. 1A). In a subsequent report, Miake *et al.* found that the I_{K1} current needed to be reduced by at least 80% in order to get cellular automaticity; lesser reductions caused a prolongation of the action potential (AP) duration with unstable repolarization.[14]

Recently, Qu *et al.* reported another method for increased myocyte automaticity.[11] In a dog model, they transferred the HCN2 gene, producing a protein thought to be responsible for I_f, the putative pacemaker current. Qu *et al.* injected solutions containing Ad-HCN2 into the left atria and noted spontaneous left atrial rhythms during vagally induced sinus arrest in four dogs (FIG. 1B). In contrast, three dogs injected with an adenovirus encoding GFP did not have any spontaneous rhythms. Subsequent work with Ad-HCN2 injection into the left bundle branch by the same group showed left bundle branch premature ventricular beats intermittently during sinus rhythm and left bundle escape rhythms in six of seven animals during vagal stimulation.[15] These data suggested that HCN2 overexpression could increase automaticity of either atrial or ventricular tissue.

Overall, gene transfer approaches to increase cardiac automaticity are in early stages of development. None of the published interventions completely recreates the sinus node, but all show promise. Ongoing work in this field has the potential for tremendous impact if the correct gene or combination of genes is identified to allow recreation of true pacemaker activity.

MODIFICATION OF ELECTRICAL CONDUCTION

Altering cardiac transcellular conduction could potentially be done using a number of strategies. The most obvious approach would be manipulation of gap junction

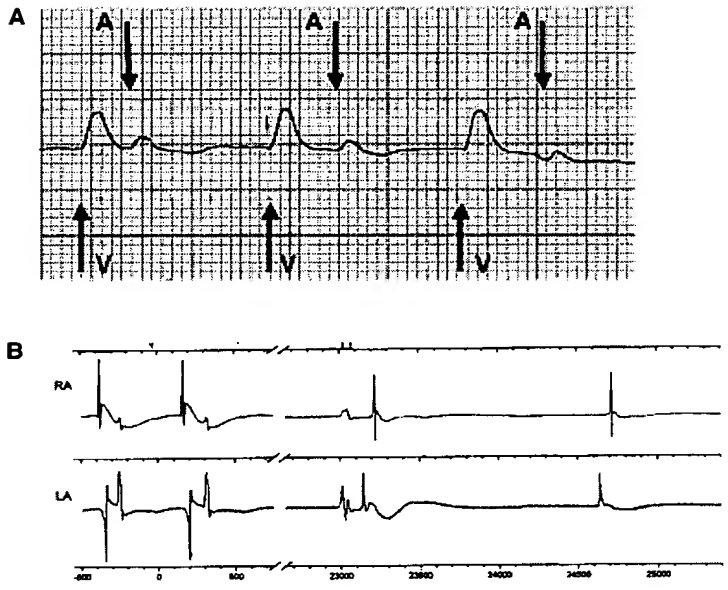

FIGURE 1. Gene transfer-induced alterations in cardiac automaticity. (**A**) Idioventricular rhythm originating in ventricular cells expressing a knock-out mutation for Kir2.1. (From Miake *et al.*[12] Reproduced by permission.) (**B**) Left atrial escape rhythm coming from cells expressing HCN2. The two beats on the *left* originate in the right atrium during normal sinus rhythm. The two beats on the *right* originate in the left atrium, from the gene transfer region, during vagally mediated sinus arrest. (From Qu *et al.*[11] Reproduced by permission.)

behavior by up- or down-regulation of connexins. In the specialized conducting regions of the heart, calcium channel alterations would affect conduction by slowing the rate of cellular activation. Similar alterations could be achieved in atrial or ventricular myocytes by manipulating sodium channel function. Manipulation of the extracellular matrix or of the distribution of gap junctions and ion channels also has potential for modification of electrical conduction, albeit at a much higher level of complexity than the other simpler manipulations.

To date, the primary strategy chosen for modifying intracardiac conduction has been manipulation of the G protein signaling cascade, which has a presumed downstream effect of slowing conduction through the L-type calcium channel. We initially achieved this effect by gene transfer of adenoviral vectors encoding wild-type $G\alpha_{i2}$ in a porcine model of acute atrial fibrillation. The viruses were delivered to the atrioventricular (AV) node by perfusion of the AV nodal artery after treatment with VEGF and nitroglycerin to increase vascular permeability to the viruses.[16,17] Control experiments with adenoviruses encoding the β-galactosidase reporter gene documented gene transfer to almost 50% of cells in the AV node.[18] AV nodal function was not affected by gene transfer with the control virus. After gene transfer of $G\alpha_{i2}$, conduction slowing and refractory period prolongation were observed in the AV node.

The ventricular response rate to acutely induced atrial fibrillation (AF) was decreased by 20% at baseline, with a persistent 15% reduction after administration of 1 mg of epinephrine (FIG. 2A). Subsequent work in a model of persistent AF and severe congestive heart failure[19] found that heart rate control was overdriven by the waking state when the wild type $G\alpha_{i2}$ was used, but a constitutively active mutant $G\alpha_{i2}$ Q205L was capable of sustained reductions in heart rate (FIG. 2B).[20] The per-

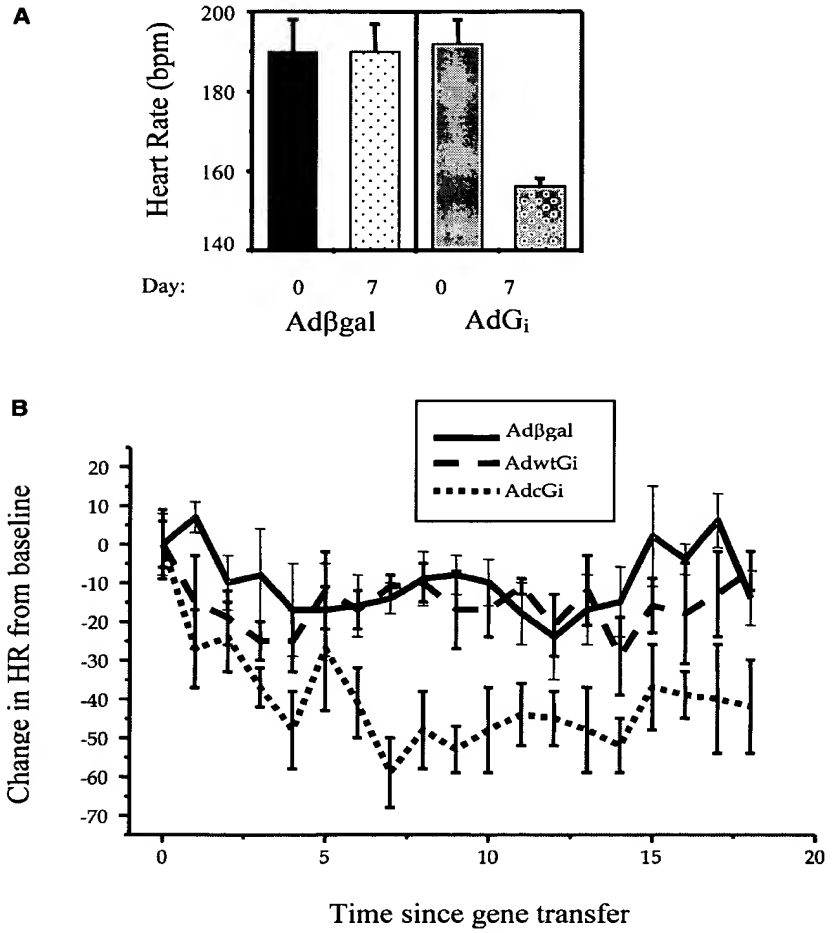

FIGURE 2. Effects of $G\alpha_{i2}$ overexpression in porcine AV node. (**A**) Percentage change in atrial fibrillation (AF) heart rate during acute atrial fibrillation. (Adapted with permission from Donahue *et al.*[18]) (**B**) Change in ventricular rate in persistent AF after gene transfer of β-galactosidase, wild type $G\alpha_{i2}$, and a constitutively active mutant $G\alpha_{i2}$ Q205L. (From Bauer *et al.*[20] Reproduced by permission.)

sistent AF model had an underlying tachycardiomyopathy caused by the uncontrolled heart rate in AF. The 20% reduction in heart rate achieved by gene transfer of the constitutively active mutant was sufficient to reverse the tachycardiomyopathy.

GENE TRANSFER EFFECTS ON CARDIAC REPOLARIZATION

Some of the earliest work in myocardial gene transfer evaluated the effects of potassium channel expression in primary cultures of ventricular myocytes. The first reported alteration of the action potential morphology by *in vitro* gene transfer used an adenovirus expressing the Shaker potassium channel, an ion channel expressed in Drosophila that causes an I_{to1}-like current.[21] Expression of Shaker in canine ventricular myocytes caused numerous changes in the AP, including amplification of phase 1, suppression of phase 2, and abbreviation of the overall AP duration. The level of expression correlated with the extent of changes in the AP, with more robustly expressing cells having the most bizarre AP morphologies.[22] Subsequent work by Nuss *et al.* showed that overexpression of HERG also shortened the AP duration but with a more physiological AP morphology.[23] HERG channels are the major component of the I_{Kr} current, and as such are activated later than the I_{to}-like current caused by the Shaker channel. This later activation allowed a normal AP notch and plateau but still abbreviated the AP duration.

Two *in vitro* reports have evaluated the therapeutic possibilities of cardiac myocyte gene transfer. Ennis *et al.* transferred a bicistronic construct encoding both a sarcoplasmic reticulum calcium adenosine triphosphate (SERCA-1) and the Kv2.1 channel responsible, in part, for the I_{K1} current.[24] They found that I_{K1} overexpression shortened AP duration and SERCA-1 overexpression increased the efficiency of intracellular calcium handling. This work suggested that combination gene therapy might be effective in heart failure, where the potassium channel overexpression could have an antiarrhythmic effect by shortening repolarization, and the SERCA overexpression could have a direct increase in contractility or in the least could obviate any negative effects on contractility from the AP shortening caused by the potassium channel overexpression. Kodirov *et al.* were able to regenerate potassium current in myocytes from Kv1.1 knock-out transgenic mice by overexpression of Kv1.5.[25] This gene-switching strategy illustrated the ability to overcome the dominant negative effects of one mutation by overexpressing a non-interacting protein of similar function.

In a recent publication, we reported a method for transmural atrial gene transfer that involved painting viral vectors complexed in poloxamer gels onto the atrial epicardium.[26] The inclusion of trypsin at low concentrations in the gel matrix allows penetration of the virus across the atrial myocardium without structural damage to the heart (FIG. 3A). Using this vector delivery method, we found that transfer of a dominant negative mutation of the HERG channel caused prolongation of atrial repolarization without affecting ventricular cells (FIG. 3B).

PROBLEMS LIMITING THE CLINICAL UTILITY OF GENE TRANSFER

Translation of arrhythmia gene therapy to clinical practice will require solutions to a number of problems that face the field, including those related to homogeneous

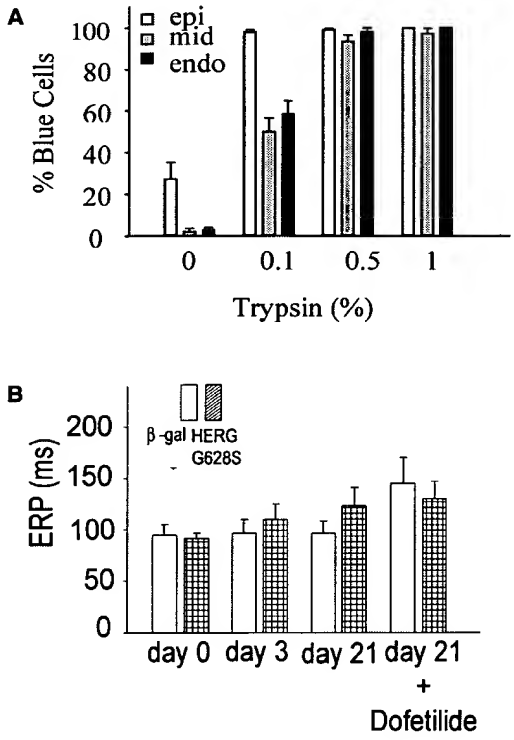

FIGURE 3. Atrial gene transfer using a newly described epicardial painting method. (**A**) Percentage of myocytes expressing the reporter gene after epicardial application of 5×10^9 pfu adenovirus in 5 ml of a gel-matrix with the given concentration of trypsin. (**B**) Effect of gene transfer on the effective refractory period (ERP) after painting atria with either Adβgal or AdHERG-G628S. βgal encodes the lacZ reporter gene. HERG-G628S is a dominant negative mutant of the HERG potassium channel. Day 21 effects are shown in the absence and presence of dofetilide. (From Kikuchi *et al.*[26] Reproduced by permission.)

delivery of the vector to the target tissue, control of gene expression, evaluation of potentially toxic effects of the vector or the transgene, and control of nontarget organ gene transfer and of host immune responses. Efforts to solve these problems are taking place on multiple fronts. Recent advances in vector design have documented the ability of adeno-associated virus and helper-dependent adenovirus vectors to sustain long-term gene expression and to reduce host immune responses.[27,28] Discovery of methods to increase microvascular permeability to vectors and to improve physical parameters relevant to gene transfer efficiency have improved efficacy and homogeneity of gene delivery.[16,17,29–31] New situation-specific promoters or response elements that activate in response to hypoxia, temperature, and steroid or drug exposure have been identified,[32] lending hope to the possibility that the timing and amount of gene expression can be controlled.

SUMMARY

This review has illustrated several ways that gene transfer techniques can be used to explore or manipulate cellular communication and ultimately cardiac function. Relatively straightforward techniques have been employed to test hypotheses regarding

the role of growth factors, ion channels, connexins, and G proteins in cardiac myocytes. The ultimate goal of these studies is translation of animal study results to human therapies. Continuing effort to improve gene delivery, to control gene expression, and to investigate gene transfer effects in a variety of "near-human" animal models will be required to realize this goal.

ACKNOWLEDGMENTS

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Gene therapy for cardiac arrhythmias.

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Myocardial gene transfer has become a routine tool to investigate the pathophysiology of cardiac diseases, although translation of gene transfer techniques into therapeutics has not come as quickly as many had hoped. In the field of cardiac arrhythmias, there is a great need for new therapeutic options. The current work reviews the use of gene transfer to evaluate cellular electrophysiology, the application of in vivo gene transfer to treat common arrhythmias, and the current problems in the field of cardiac gene therapy. Arrhythmia gene therapy is a field in its infancy, and future human applications are dependent on solutions to the problems discussed in this review.

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1: Ann Med. 2004;36 Suppl 1:98-105.

Gene transfer techniques for cardiac arrhythmias.

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Therapy for cardiac arrhythmias is inadequate, based on current options. Gene therapy has shown tremendous potential to investigate pathophysiology and potential therapies for cardiac diseases. The current work reviews the possibilities for application of in vivo gene transfer to treatment of common arrhythmias, including vector selection, delivery technique, and data on in vivo gene transfer for rate control in atrial fibrillation and for pacemaking activity. Arrhythmia gene therapy is a field in its infancy, and future human applications are dependent on solutions to the problems discussed in this review.

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Focal modification of electrical conduction in the heart by viral gene transfer.

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Modern treatment of cardiac arrhythmias is limited to pharmacotherapy, radiofrequency ablation, or implantable devices. Antiarrhythmic medications suppress arrhythmias, but their systemic effects are often poorly tolerated and their proarrhythmic tendencies increase mortality. Radiofrequency ablation can cure only a limited number of arrhythmias. Implantable devices can be curative for bradyarrhythmias and lifesaving for tachyarrhythmias, but require a lifetime commitment to repeated procedures, are a significant expense, and may lead to severe complications. One possibility is the use of gene therapy as an antiarrhythmic strategy. As an initial attempt to explore this option, we focused on genetic modification of the atrioventricular node. First, we developed an intracoronary perfusion model for gene delivery, building on our previous work in isolated cardiac myocytes and hearts perfused ex vivo. Using this method, we infected porcine hearts with Adbetagal (recombinant adenovirus expressing Escherichia coli beta-galactosidase) or with AdGi (adenovirus encoding the Galphai2 subunit). We hypothesized that excess Galphai2 would mimic the effects of beta-adreneric antagonists, in effect creating a localized beta-blockade. Galphai2 overexpression suppressed baseline atrioventricular conduction and slowed the heart rate during atrial fibrillation without producing complete heart block. In contrast, expression of the reporter gene beta-galactosidase had no electrophysiological effects. Our results demonstrate the feasibility of using myocardial gene transfer strategies to treat common arrhythmias.

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POINT OF VIEW

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Somatic Gene Transfer and Cardiac Arrhythmias: Problems and Prospects

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Introduction

Coronary artery disease and cardiomyopathy are the principal substrates in which the majority of life-threatening cardiac arrhythmias exist. Gene therapy is becoming a viable alternative and adjunct to more conventional therapies for coronary artery disease and cardiomyopathy.[1] Cardiac arrhythmias are a promising but less well-developed target for gene-based therapeutic strategies. Regardless of the disease target, a number of considerations will directly impact the risk of arrhythmias with any form of gene-based therapy in the heart. This Point-of-View addresses the rationale and mechanics of gene-based therapy in structural heart disease, the prospects for therapies targeting cardiac arrhythmias, and cautions regarding this exciting therapeutic approach.

Somatic Gene Transfer, Gene Therapy, and Cell Therapy

Somatic gene transfer refers to the addition of genes (as either DNA or RNA) to cells other than eggs or sperm, and *gene therapy* entails gene transfer to a target somatic cell or organ to either treat or prevent a disease. The types of delivery systems vary from naked plasmid DNA to DNA in chemical carriers (e.g., liposomes) or foreign DNA inserted into a viral genome. Gene transfer to the heart may occur in other ways, such as the use of stem cells or other myogenic progenitor cells that are exogenous or endogenous to the organism (for review see reference 2). Finally, the genes delivered to the heart need not encode a protein but may reduce or eliminate protein function through the delivery of antisense oligonucleotides or ribozymes that interfere with protein transcription and translation.[3,4]

A central mandate of gene therapy is the successful delivery of the nucleic acid to the target tissue. A number of gene delivery systems have been developed that utilize viral- and nonviral-based methods; the latter includes both physical and chemical agents, as well as cell-based therapy. Vectors used in gene therapy are the subject of numerous reviews (e.g., reference 5) and will only briefly be reviewed. In many ways, viruses are ideal vectors because they have evolved the ability to efficiently deliver nucleic acid (i.e., viral genome) and avoid immune surveillance. Each of the viral vector systems has its advantages and disadvantages.

The most used viral vectors in clinical gene therapy trials are the retroviruses. These small RNA viruses often require dividing cells for entry into the cell nucleus and integration into the cell genome. The integration event affords expression of the gene product for the life of the cell. The requirement for dividing cells is not absolute. Certain retroviruses, such as the lentiviruses [e.g., human immunodeficiency virus (HIV)], can infect and integrate into the genome of nondividing cells. The integration into the genome has the potential for disruption of essential genes and malignant transformation. Retroviral vectors are commonly used in cancer gene therapy and often for ex vivo infection of isolated cells that can be propagated in culture. Disadvantages of retroviral vector systems include the relatively short half-life of the viruses, difficulty in manufacturing the concentrations necessary for in vivo myocardial or vascular gene transfer, the need for actively dividing target cells (with the exception of lentiviruses), and public reluctance toward any therapy using an HIV-like system.

Replication-deficient adenoviruses are another workhorse vector and the most commonly used for cardiovascular applications.[1] These viruses have the advantage of being able to infect nondividing cells and not integrating into the genome. This results in the disadvantage of transient expression of the gene product. In addition, both cell-mediated and humoral immune responses limit gene expression.[5] Adeno-associated virus (AAV) is a single-stranded DNA virus of the parvovirus family that requires a helper virus for replication. The advantages of AAV as a gene therapy vector are its ability to infect nondividing cells, integrate into the host genome, and evade the host immune system, thus producing efficient and long-term gene expression.[6] Disadvantages include the requirement for coinfection with a helper virus, such as adenovirus or herpes simplex virus (HSV), and the limited size of genes that can be packaged.[7] Another disadvantage of AAV vectors is the complex process required for virus production, with requirement for helper viruses or plasmids to accomplish virus amplification. Other viruses that have been used or are in development as gene therapy vectors include HSV, RNA viruses such as polio, hepatitis A, and Sindbis viruses.[5]

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Nonviral gene delivery systems use physicochemical methods to facilitate gene transfer, such as liposome-mediated delivery, gene guns, and DNA conjugates to polycations. In general, methods that involve the delivery of DNA alone either by physical or chemical methods are less pathogenic but also less efficient in genetic transduction of most tissues, including the heart. The advantages of nonviral vectors are their relative ease of manufacturing and possibly their greater public acceptance (e.g., when compared to lentiviruses). Nonviral vectors share many of the viral vector problems with longevity of expression and incitement of an immune response. The efficiencies of these methods are variable but tend to be less than that of viral vectors; however, they avoid any of the complications associated with integration of genes into the host genome.

Cell therapy is an indirect means of delivering new genes and proteins to the heart. Experimental studies have demonstrated the feasibility of engraftment of skeletal muscle myoblasts into the heart.[8] Engrafted myoblasts improve left ventricular performance, although systolic function of the exogenous cells is uncertain.[2] These cells do, however, appear to form gap junctions with cardiac myocytes in animal models.[9] The feasibility of myoblast transplantation has been demonstrated in humans.[10] The electrophysiologic implications of cell therapy in the human heart have yet to be determined.

Human Clinical Trials

The first gene therapy trial in 1990 ushered in an era of excitement and in some cases profound disappointment (for review see reference 1). The initial disease targets were monogenic disorders (e.g., adenosine deaminase deficiency, cystic fibrosis) in organs that were easily accessible (bone marrow and lung). Currently there are more than 635 clinical trials in gene therapy worldwide, of which 70% are for cancer and related illnesses, but cardiovascular disease constitutes a significant minority of clinical trials *(http://www.wiley.co.uk/genetherapy/clinical/)*. The principal disease targets for gene therapy in the cardiovascular system are atherosclerosis, including peripheral vascular disease, and heart failure. There is only a single report in the literature of in vivo utilization of gene therapy to specifically target an arrhythmia[11] and a limited number of reports using in vitro gene transfer to investigate arrhythmia mechanisms on a cellular level. Only therapeutic applications in atherosclerosis have been used in human studies. A detailed listing of previous and current clinical gene transfer trials can be obtained at *http://www4.od.nih.gov/oba/rac/clinicaltrial.htm*.

For treatment of atherosclerotic cardiovascular disease, Isner and colleagues have championed the notion that exogenous angiogenic growth factors could promote revascularization of ischemic tissues, an approach that they have referred to as *therapeutic angiogenesis*. The viability and stability of these newly formed vessels has been questioned, but uncontrolled studies have shown an improvement of symptoms in patients with intractable angina or peripheral vascular disease.[1]

The molecular targets in heart failure, particularly polygenic forms, are less certain but include proteins involved in calcium homeostasis, adrenergic receptors and their modulators, and mediators of programmed cell death or apoptosis. A conceivable list of targets applicable to gene therapy for arrhythmias could include molecules that alter active membrane properties, such as ion channels and transporters, or those that mediate cell-to-cell communication (i.e., connexins).

Gene transfer to the heart presents substantial technical challenges to gene delivery and durable expression of the encoded gene products. There are also unique complications to the introduction of genes that intentionally or incidentally modify cardiac excitability. In contrast to other therapeutic applications, gene therapy in the cardiovascular system to date has disproportionately used naked DNA, particularly for atherosclerotic cardiovascular disease. This is possible because the genes that are transferred [vascular endothelial growth factor (VEGF) or fibroblast growth factor) need not transform a large number of cells. Instead, VEGF[1] and fibroblast growth factor[12] are naturally or genetically engineered to be secreted so that a small number of transduced cells that secrete these angiogenic factors influence the biologic activity of a larger number of target cells. Gene therapy for treatment of heart failure is not likely to have this luxury, and efficient gene transfer to a substantial portion of the myocytes in the failing heart may be necessary. In contrast, certain arrhythmias may be amenable to the focal delivery of genes (e.g., scar-mediated tachycardia and modification of the AV node[11]), making paracrine transduction of other target cells unnecessary.

Molecular Targets for Arrhythmias

There are a number of both focal and global targets for gene therapy of cardiac arrhythmias. The most straightforward is the replacement or correction of an abnormal gene in monogenic disorders of cardiac rhythm, such as the long QT syndrome (LQTS), and idiopathic ventricular tachycardias and fibrillation (Brugada syndrome or catecholaminergic polymorphic ventricular tachycardia). Similarly, correction of single gene defects that produce structural heart disease may be antiarrhythmic. Most frequently, however, the target will be polygenic structural heart disease that forms the substrate for arrhythmias (scar-mediated ventricular tachycardia, atrial fibrillation in diseased atria). Another strategy may be to focally modify the electrophysiology of the myocardium, for example, in the AV node or a zone of slow conduction that is mediating reentry. In either case, substantial hurdles to clinical implementation will have to be negotiated.

An understanding of the basic principles of cellular electrophysiology can be used to target specific genes that will modify active membrane properties of the cardiac myocyte. Proof of principle has been demonstrated in a number of in vitro experimental models with a variety of delivered genes. Studies in isolated cardiac ventricular myocytes demonstrated the ability to alter the action potential (AP) profile by infection with a channel gene-containing virus. For example, reconstitution of the transient outward current (I_{to}), which is down-regulated in the failing heart (for review see Tomaselli and Marban[13]), can shorten the ventricular AP duration. However, a number of limitations of this approach were immediately apparent, for example, overexpression of Kv4.3, the gene that encodes I_{to}, produced a bizarre and overly shortened AP.[14] Overexpression of the gene product of *HERG* (the disease gene in LQTS2), I_{Kr}, produced a more normal appearing AP, because the activation kinetics of I_{Kr} are slower than I_{to}, thus avoiding short-circuiting the AP plateau.[15] The in vitro experiments raise a series of questions that must be

addressed prior to implementation of gene therapy that targets the AP profile. First, fine control of the regulation of the level of channel proteins is still not feasible with strong constitutive promoters that are common to viral gene constructs but may be improved with the use of inducible promoters. Although the AP profile can be changed by any number of ion channel manipulations, the detailed cellular electrophysiology will depend on the channel gene that is introduced (or eliminated), and it may be difficult to predict the organ-level electrophysiologic consequences of such changes in the myocyte. Finally, the density of ionic currents and expression of ion channel genes and proteins vary regionally in the heart. Controlled, regionally specific expression is unlikely with currently available technology. Correction of ion channel remodeling in the structurally diseased heart will be more feasible when the upstream regulation of ion channel expression is better understood.

A number of challenges confront gene therapy as a method to correct monogenic diseases of the heart. First, correction of monogenic ion channel (and other gene) defects will require genomic replacement of the mutant allele(s) and often elimination of the mutant allele, particularly if it exhibits abnormal function or dominant negative suppression of the normal allele. Simple replacement of the mutant ion channel genes may be insufficient when one considers the regional heterogeneity of ionic currents in the heart. Again, understanding the regulation of expression of these genes is a necessary first step to the design of rational gene replacement therapy.

Focal modification of cardiac electrophysiology has been demonstrated in principle. In the only in vivo report of gene transfer-mediated treatment of an arrhythmia, conduction through the AV node was targeted. In a porcine model of acute atrial fibrillation, adenoviruses containing the inhibitory G-protein $G\alpha_{i2}$ (AdGi) were infused into the AV nodal artery. Adβgal, an adenovirus encoding *Escherichia coli* β-galactosidase, was used as a reporter to document the extent of gene transfer, and it was used as a control to identify nonspecific effects of adenovirus infection and gene transfer in the AV node.[11] Prior to virus perfusion, VEGF and nitroglycerin were infused into the AV nodal artery to increase the efficiency of gene transfer.[16] Adβgal-infected hearts showed gene transfer to 45% of AV nodal myocytes and no effect on AV nodal function. Several days after infection, a limited mononuclear inflammatory infiltrate was noted. AdGi-infection slowed conduction through the AV node, ultimately leading to a reduction in the heart rate during acutely induced atrial fibrillation.

Another gene therapy strategy to alter conduction is manipulation of heart or progenitor cells ex vivo, creating designer myocytes or specialized conducting tissue cells to modify cardiac excitability. A recent example is the creation of "automatic" ventricular myocytes that may serve as biologic pacemakers.[17] The strategy was to suppress the inward rectifier current (I_{K1}) with a dominant negative Kir2.1 gene construct that assembles with normal channel subunits but acts as a "poison pill" for the macromolecular complex such that the channel does not conduct K^+ current. This is an example of gain of function by gene transfer-mediated suppression of an endogenous ion channel. The use of cell therapy circumvents some of the problems related to delivery of genes to individual cells and problems related to malignant transformation of cells as a consequence of integration of gene products. However, concerns regarding delivery of the transduced cells to the heart, as well as their full integration into the myocardium, are significant obstacles to their use as a therapeutic strategy.

Challenges to Gene Therapy for Treatment of Arrhythmias

Direct and indirect gene transfer is an important tool in the study of normal and pathologic cardiac electrophysiology. The use of gene transfer in clinical therapeutics remains intellectually appealing but is subject to a number of substantial challenges before implementation in humans can be considered. These challenges are relevant to gene therapy generically and to the treatment of cardiac arrhythmias specifically.

The conventional problems in gene therapy are related to homogeneity of delivery of the vector to the target tissue, regulation of gene expression, potentially toxic effects of the vector or the transgene, nontarget organ gene delivery, and host immune responses. Efforts to solve these problems are taking place on multiple fronts. Recent advances in vector design have demonstrated the ability of adeno-associated virus and helper-dependent adenovirus vectors to sustain long-term gene expression in the heart and to reduce host immune responses.[6,18] Methods that increase physical contact between vector and target cells have improved efficacy and homogeneity of gene delivery.[16,19] New situation-specific promoters or response elements that activate in response to hypoxia, temperature, and steroid or drug exposure have been used[20] in the hope that the timing and amount of gene expression can be controlled.

Problems that are more specific to gene therapy for cardiac arrhythmias are exemplified by, but not limited to, our lack of understanding of the molecular mechanisms of many arrhythmias and the spatial complexity of expression of ion channels, which curbs the utility of transfer of a single ion channel species. In the long term, it likely will be better to alter the regulation of channel expression by modulating the native promoters of those channels. Focal approaches to the delivery of genes or cells must confront the problems of accurate site selection and efficient and durable transduction of cells and/or the integration of exogenous cells into the cardiac syncytium. The potential rewards of gene/cell therapy for cardiac arrhythmias are great; however, substantial challenges remain prior to implementation of such therapy in humans.

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